Cytoglobin Expression Is Induced In Vascular Smooth Muscle Cells Through Endothelial Cell-derived Notch Signaling:

Implications of a Protective Role in Blood Vessel Function

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Introduction

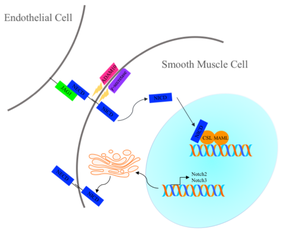
Cardiovascular Disease is the leading global cause of death, claiming upwards of 17.3 million lives each year

- Vascular diseases exhibit defects in blood vessel formation and function
- Modeling vascular defects on the molecular level can provide insight into the key mechanisms of disease progression and identify novel avenues for intervention

Our Mission is to understand the molecular signaling pathways that govern cell-cell communication in relation to vascular structure and function

Notch Signaling is implicated in angiogenic re-modeling, arterial/ venous specification and tip cell differentiation

- Requires cell to cell contact – signal is passed from ligand (Jag 1-2, DLL 1, 3, 4) on sending cell to receptor on receiving cell (Notch 1-4)
- Ligand binding results in cleavage of the Notch Intracellular Domain (NICD), which translocates to the nucleus and serves as a transcription factor



Cytoglobin is a ubiquitously expressed hexa-coordinate hemoglobin molecule that binds O_2, CO, and NO

- Putative roles include protecting against hypoxia and oxidative stress and maintaining vascular homeostasis
- Implicated in the modulation of nitric oxide and reactive oxygen species

Nitric Oxide serves to maintain vascular homeostasis by modulating vascular dilator tone, regulating cell growth, and protecting the vessel from circulating molecules

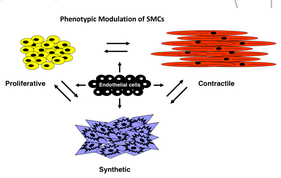
Reactive Oxygen Species (ROS) are a byproduct of cellular stress and contribute to vascular disease

Background

Cytoglobin was identified in a previous Lilly Lab study as a gene that is induced by co-culture

Notch Signaling is a critical mediator of Endothelial Cell (EC) – Smooth Muscle Cell (SMC) dependent communication and resulting gene expression profiles

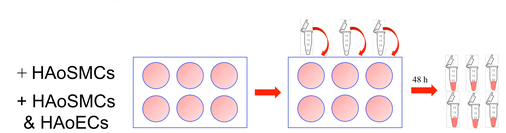
- ECs activate Notch signaling in adjacent SMCs to induce gene expression profiles, which contribute to SMC function
- Critical cell – cell contact is modeled by co-culturing ECs and SMCs



Hypothesis: *Cytoglobin expression is induced in SMCs by endothelial derived Notch signaling and regulates nitric oxide bioavailability in blood vessels*

Methods

- *Cell Culture.* Primary cultured Human Aortic Smooth Muscle Cells (HAoSMCs) and Human Aortic Endothelial Cells (HAoECs) were purchased. Cells were cultured in EBM-2 whether cultured alone or together. For co-culture, 6 x 10^4 SMCs cells were plated in a 12 well dish with 6 x 10^4 ECs and cultured for 48 hr, and various treatments were introduced.
- *Treatments.* Cell treatments included γ-secretase inhibitor DAPT, Notch2 siRNA, Notch3 siRNA, CYGB siRNA, NO Donor, CPITO, H_2O_2, and $CoCl_2$.
- *Sample Processing.* When separated, HAoSMCs were isolated using anti-platelet endothelial cell adhesion molecule conjugated Dynabeads. RNA transcript levels were compared using qPCR and protein levels were examined using Western Blots.



Results

CYGB is Induced in SMCs by Co-cultured ECs

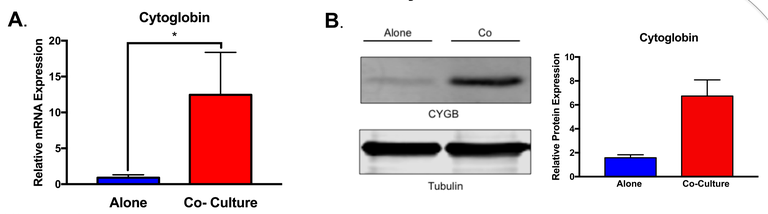


Figure 1. CYGB expression in SMCs in response to co-culture with ECs. (A) RNA was isolated from HAoSMCs that were cultured either alone or in co-culture. qPCR was performed in order to determine the relative expression of RNA transcripts. * P < 0.01 (B) Protein lysate was isolated from HAoSMCs that were cultured either alone or in co-culture. A Western Blot was performed in order to examine relative protein expression.

CYGB is Subject to Regulation by the Notch Signaling Pathway

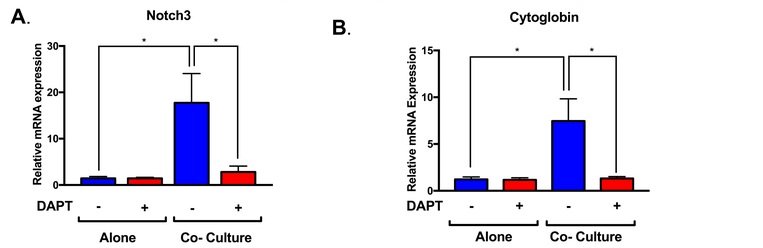


Figure 2. CYGB expression when treated with DAPT. RNA was isolated from HAoSMCs that were cultured either alone or in co-culture with HAoECs and in the presence or absence of DAPT. Notch3 is a known target of the Notch signaling pathway and served as a positive control. qPCR was performed in order to determine the relative expression of (A) Notch 3 and (B) CYGB RNA transcripts.
* P < 0.01

CYGB is Uniquely Regulated by the Notch2 Receptor

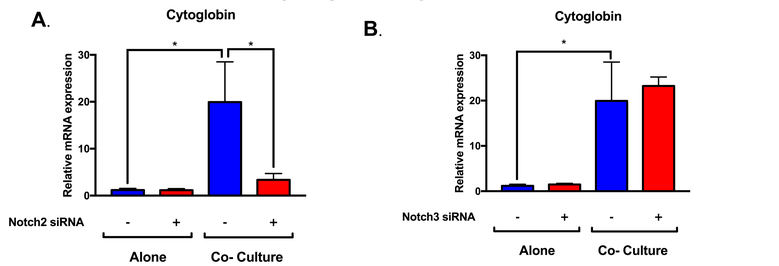


Figure 3. CYGB expression when treated with siRNA. RNA was isolated from HAoSMCs that were cultured either alone or in co-culture with HAoECs and with control, Notch2 (A) or Notch3 (B) siRNA. qPCR was performed in order to determine the relative expression of CYGB RNA transcripts.
* P < 0.01

Knockdown of CYGB Expression by siRNA

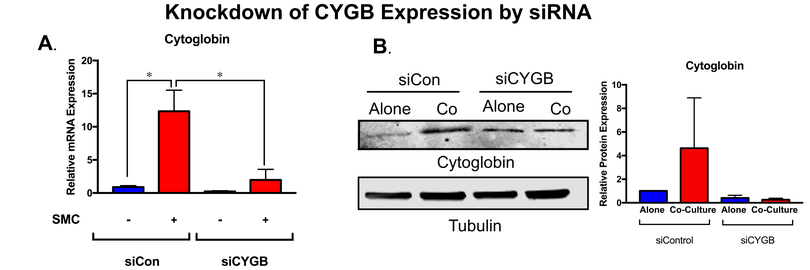


Figure 4. (A) RNA was isolated from HAoSMCs that were cultured either alone or in co-culture and with either control or CYGB siRNA. Transcript levels were measured via qPCR. * P < 0.01
(B) Protein lysate was isolated from HAoSMCs that were cultured either alone or in co-culture and with control or CYGB siRNA, and a Western Blot was performed.

NADPH Oxidase 4 Expression Increases in the Absence of CYGB in Co-Culture

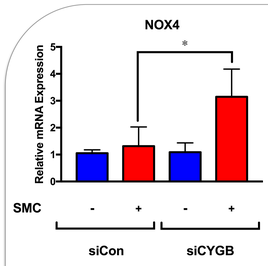


Figure 4. RNA was isolated from HAoSMCs that were cultured either alone or in co-culture and with control or CYGB siRNA. NADPH Oxidase 4 (NOX4) expression was evaluated via qPCR. * P < 0.01

CYGB Expression is Influenced by ROS and Hypoxia Mimics

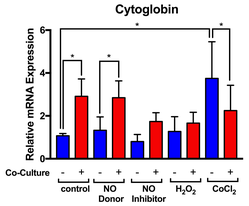


Figure 2. RNA was isolated from HAoSMCs that were cultured either alone or in co-culture and in the presence or absence of various agents. CYGB RNA transcripts were evaluated via qPCR. * P < 0.01

Conclusions

- CYGB is up-regulated in co-culture conditions as demonstrated by relative mRNA transcript levels and CYGB protein levels
  - → *CYGB is a gene induced in SMCs by co-cultured ECs*
- Chemical inhibition of Notch signaling blocks induction of CYGB in co-culture
  - → *CYGB is subject to regulation by the Notch signaling pathway*
- Transfection with Notch2 specific siRNA inhibits the induction of CYGB expression
  - → *Notch signaling is necessary to induce CYGB expression*
- Transfection with Notch3 specific siRNA had no statistically significant effect on the induction of CYGB expression
  - → *CYGB may be uniquely regulated by the Notch2 receptor in SMCs*
- CYGB knockdown results in increased expression of NOX4 in SMCs
  - → *CYGB likely contributes to ROS modulation*
- CYGB expression is altered by ROS and activation of hypoxia-induced pathways
  - → *Alteration of CYGB expression will be considered during functional analysis*

Future Directions

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Functional Assays. Utilize siRNA knockdown of CYGB expression to block function

- Examine mitochondrial superoxide levels, ROS levels, nitrite formation, and cyclic guanylyl monocyclase levels using a variety of assays

Significance

- Examining the function of CYGB in SMCs indicates a link to managing vascular derived stress, and CYGB may provide the means for which SMCs can regulate nitric oxide bioavailability in blood vessels
- Further examination of Notch signaling and CYGB expression in dysfunctional vessels may reveal novel targets for therapeutic intervention

References

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Acknowledgements

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